

of glucose. Alanine (10mM) or arginine(20mM) were added, and the cells were incubated for 90 minutes, at which time samples were collected for assay of insulin content. The results are shown in Table 13.

5

Table 13

Effect of amino acids on glucose stimulated insulin secretion.

	total mM glucose	control	+10 mM alanine	+20 mM arginine
10	1 mM	91	103	128
	2 mM	108	135	134
	6 mM	112	127	105
	11 mM	116	117	93
	16 mM	128	101	99
	22 mM	115	87	94

- 15 Results: At low glucose concentrations (1, 2, and 6 mM), alanine increased insulin secretion beyond the level of glucose stimulation alone. The effect of alanine was most pronounced at 2 mM glucose, where alanine increased insulin secretion 1.25 fold over that stimulated by glucose alone.
- 20 Arginine had a pronounced effect at 1 mM glucose, where arginine increased insulin secretion 1.4 fold over that stimulated by glucose alone.

#### EXAMPLE 21

This example demonstrates that human pancreas cells maintained 25 in long-term culture contain immunoreactive insulin.

Human pancreas cells from Example 16, passage generation 47, were fixed and permeabilized by -20°C methanol, mounted, and stained by a standard immunochemical technique (Harlow, E. et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratories) using as a primary antibody an anti-human insulin antibody raised in guinea pig from Peninsula Laboratories, Belmont, California. The secondary antibody was anti-guinea pig IgG (whole molecule) - TRITC conjugate (Rb) from Sigma (T-7153). As a negative control, in place

of the primary antibody, cells were incubated with the same primary anti-insulin antiserum which had been pre-incubated with synthetic human insulin to adsorb the anti-insulin antibodies. The cells were counterstained with Hoechst dye 5 33258. The immunostained cells were observed and photographed on a Zeiss IM35 microscope using a Zeiss #15 filter to illuminate the rhodamine dye labeling for insulin. The identical cell fields were observed and photographed using a Zeiss #2 filter to illuminate the Hoechst labeling of DNA 10 in the nuclei of all cells in the field. Photographs of cell nuclei were compared with counterpart photographs of rhodamine labeled cell cytoplasms to determine how many cells in the field contained immunoreactive insulin.

Results: Controls showed no background staining. Comparison 15 of numbers of labeled nuclei with numbers of cells immunoreactive to insulin revealed that greater than 60% of the cells in the culture contained immunoreactive insulin at different intensities of fluorescent staining.

WHAT IS CLAIMED IS:

1. A method for establishing hormone-secreting cells in vitro comprising the steps of:
  - 5 a) selecting at least one cell having hormone-secreting potential from a population of similar cells having hormone-secreting potential,
  - b) placing said cell in an establishing medium, said establishing medium being capable of promoting the viability
  - 10 of said cell for at least about 13 days in vitro, and
  - c) maintaining the viability of at least one of said cell and the progeny of said cell for at least about 13 days in vitro.
2. The method according to claim 1 wherein,
  - 15 said cells are selected to have at least two characteristics selected from the group consisting of:
    - (a) a smooth outer membrane,
    - (b) one of an approximately spherical shape and a substantially ovoid shape ,
    - 20 (c) a non-granular cytoplasm, and
    - (d) being a member of a clump of cells numbering from about 2 to about 12 cells and being approximately homogeneous in size and shape.
3. The method according to claim 1 comprising the further
  - 25 step of subdividing said progeny into a plurality of cell cultures.
4. The method of claim 2 wherein,
  - said selection is accomplished by visual observation through a microscope, and
  - 30 further comprising the step of aspiration of at least one of an individual cell and cell clumps.
5. The method of claim 1 wherein,
  - said method is centrifuge-free.

6. The method of claim 5 wherein,  
said method is substantially enzyme-free.
7. The method of claim 1 and,  
prior to step (a), placing a group of cells including  
5 said population of similar cells in a solution having  
substantially the same chemical composition as the biological  
fluid which naturally surrounded said group of cells in vivo,  
the concentration of said group of cells within said solution  
being less than or equal to the concentration of cells  
10 occurring within said biological fluid, and  
prior to step (a), separating said population of cells  
from said group of cells in said solution by selecting cells  
having characteristics of viable cells and removing said cells  
from said solution.
- 15 8. The method of claim 7 wherein,  
said step of selecting cells having characteristics of  
viable cells is accomplished by selecting cells having  
characteristics selected from the group consisting of:
  - (a) cells having a smooth plasma membrane,
  - 20 (b) cells spreading in a monolayer on the bottom of the  
culture dish, and
  - (c) cells free from blood clots.
9. The method of one of claims 1, 2, 3, and 8 wherein,  
said step (b) of claim 1 is accomplished by placing said  
25 cell in an establishing medium having an osmolarity of about  
248 mOsm to about 300 mOsm.
10. The method of claim 9 wherein,  
said step (b) is accomplished by placing said cell in  
an establishing medium further having:
  - 30 (i) a basal medium comprising essential minerals,  
salts, vitamins, amino acids, and lipids,
  - (ii) a buffering system,
  - (iii) glutamine in the amount of about 6.35 mM to about  
8.35 mM, and

(iv) at least one energy source selected from the group consisting of lactate and pyruvate.

11. The method of claim 9 wherein,  
said placing step is accomplished by placing said cell  
5 in an establishing medium further having a serum in the amount  
of about 0.5% to about 15% of the total volume of the medium.
12. The method of claim 9 wherein,  
said placing step is accomplished by placing said cell  
in an establishing medium further having a serum substitute  
10 in the amount of about 5% to about 15% of the total volume  
of the medium.
13. The method of claim 11 wherein,  
said placing step is accomplished by placing said cell  
in an establishing medium having human serum.
- 15 14. The method of claim 13 and,  
the additional step of obtaining said serum from the  
blood of a donor of said cell.
15. The method of claim 9 wherein,  
said step (b) is accomplished by placing said cell in  
20 an establishing medium further having mammalian serum protein  
in the amount of about 0.5% to about 3.0% (w/v).
16. The method of claim 15 wherein,  
said placing step is accomplished by placing said cell  
in an establishing medium having bovine serum albumin.
- 25 17. A cell culture produced according to the method of claim  
9.
18. A cell culture produced according to the method of claim  
9 wherein,

said population of cells is derived from a tissue selected from the group consisting of ovary, endometrium, pituitary, thyroid, and pancreas.

19. A cell culture produced according to the method of claim  
5 9 wherein,

said population of cells is derived from a non-tumorous tissue.

20. A method for the long-term maintenance of hormone-secreting cells in vitro comprising the steps of:

10 a) placing at least one cell in a defined culture medium having an osmolarity of about 248 mOsm to about 300 mOsm, said cell having hormone-secreting potential and said cell being capable of proliferating in said medium to form progeny, and

15 b) propagating the progeny of said cell, said defined culture medium being capable of promoting the viability of at least some of the progeny of said cell.

21. The method of claim 20, and  
prior to step (a), obtaining said cell from a tissue  
20 selected from the group consisting of ovary, endometrium, trophoblast, pituitary, thyroid, and pancreas.

22. The method of claim 20 wherein,  
said placing step is accomplished by placing a cell in  
said defined culture medium which will produce progeny having  
25 potential for secretion of at least one hormone belonging to  
the group consisting of estrogens, progestins, follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotrophin, thyroxin, glucagon, and insulin.

23. The method of claim 20 wherein,  
30 said step (a) is accomplished by placing said cell in  
a culture medium further comprising:  
(i) a basal medium containing essential minerals, salts, vitamins, amino acids and lipids,

- (ii) a buffering system,
  - (iii) protein,
  - (iv) at least one energy source selected from the group consisting of lactate and pyruvate, and
- 5 (v) glutamine in the amount of about 6.35 mM to about 8.35 mM.

24. The method of claim 23 wherein said culture medium further comprises a serum substitute in an amount of about 5% to about 15% of the total volume of the medium.

10 25. The method of claim 20 wherein,  
said placing step is accomplished by placing said cell  
in a defined culture medium having an osmolarity in the range  
of about 269 mOsm to about 275 mOsm.

15 26. A cell culture produced according to a method of one of  
claims 20, 21, and 22.

27. A cell culture produced according to the method of claim  
20 wherein

said cells are derived from pancreas, and  
said cells secrete a maintenance level of insulin.

20 28. A cell culture according to claim 20 wherein  
said maintenance level of insulin is about 2 uIU to about  
1000 uIU insulin/hour per  $10^5$  cells per milliliter of defined  
culture medium.

25 29. A cell culture according to claim 20 wherein  
said maintenance level of insulin is about 20  
uIU to about 400 uIU insulin/hour per  $10^5$  cells per milliliter  
of defined culture medium.

30. A method according to claim 20 further comprising:  
subsequent to said propagating step, placing said progeny  
30 in a glucose-poor medium, and thereby  
causing said cells to secrete a basal level of insulin.

31. A cell culture produced according to claim 30 wherein,  
said basal level of insulin is about 20 uIU to about 250  
uIU insulin/hour per 1.5 million cells per milliliter of  
glucose-poor medium.
- 5 32. A method according to claim 27 and,  
after said step of placing said progeny, contacting said  
cells with about 0.5 mM to about 22 mM glucose.
- 10 33. A cell culture produced according to claim 32 wherein,  
said cells are responsive to said glucose contact to  
produce increased insulin secretion in an amount of about 1.2  
fold to about 130 fold a basal level of insulin secretion,  
said basal level being in the range of about 20 uIU to about  
250 uIU insulin per 1.5 million cells per milliliter of  
medium.
- 15 34. A cell culture according to claim 33 wherein,  
said response occurs over a time period comprising about  
30 minutes to about 24 hours.
- 20 35. A method according to claim 30, and  
after said step of placing said progeny, contacting said  
progeny with about 2 mM to about 9 mM glucose.
36. A cell culture produced according to the method of claim  
35 wherein,  
said cells respond to said glucose contact to produce  
an increase in said insulin secretion in an amount in the  
25 range of about 1.5 to about 10 fold said basal level of  
insulin secretion.
37. A method according to claim 30 further comprising,  
subsequent to said placing step, contacting said cells  
with 1 mM to 6 mM glucose, thereby causing said cells to  
30 secrete an intermediate level of insulin, and  
contacting said cells with an amino acid.

38. A cell culture produced according to claim 37 wherein,  
said cells respond to said amino acid contact to produce  
an increase in insulin secretion in a range of about 1.3 to  
about 2.0 fold said intermediate level of insulin secretion.

5 39. A cell culture according to claim 38 wherein said amino  
acid is at least one of alanine and arginine.

40. A cell culture according to claim 39 wherein said amino  
acid comprises alanine in a concentration of about 10 mM.

10 41. A cell culture according to claim 39 wherein said amino  
acid comprises arginine in a concentration of about 20 mM.

42. A cell culture produced according to the method of claim  
20 wherein,

said cells are derived from a non-tumorous tissue.

43. A method for obtaining viable follicular cells comprising  
15 the steps of:

a) placing in a medium at least one ovarian follicle,  
said follicle comprising an ovum and zona radiata cells  
adhering to said ovum,

20 b) loosening said zona radiata cells from said ovum,  
and

c) stripping said zona radiata cells from said ovum.

44. The method of claim 43 wherein,

step (b) is achieved by contacting said follicle with  
sperm.

25 45. The method of claim 43 wherein,

step (c) is accomplished by aspirating from said medium  
and expelling into said medium said follicle until separation  
of said zona radiata cells from said ovum is accomplished.

30 46. The method of claim 43 further comprising placing said  
cells in an establishing medium.

47. The method of claim 46 further comprising,  
placing said cells in a defined culture medium having  
an osmolarity of about 248 to about 300 mOsm, and  
propagating the progeny of said cells.

5 48. A method for obtaining a hormone comprising the steps  
of:

- a) placing at least one cell in a defined culture medium in vitro, said cell having hormone-secreting potential and said cell being capable of proliferating in vitro to form 10 progeny,
- b) propagating the progeny of said cell, said culture medium being capable of promoting the viability of the progeny of said cell, said progeny secreting a quantity of hormone into said medium, and
- 15 c) isolating at least a portion of said quantity of hormone.

49. The method of claim 48, and the further step of:  
contacting said cell progeny with a secretagogue selected to stimulate said secretion of said hormone.

20 50. The method of claim 49 wherein,  
said contacting is accomplished by contacting said cell with a secretagogue selected from the group consisting of follicle stimulating hormone, luteinizing hormone, chorionic gonadotrophin, potassium ion, glucagon-like peptide-1, 25 glucose, cAMP and chemical analogs of cAMP.

51. A method according to claim 48 and,  
prior to step (a) pre-treating said cell in vivo by administering at least one of a hormone and hormone-analog drugs to a cell donor.

30 52. A method according to claim 51 wherein,  
said pre-treating step is accomplished by pre-treating a female donor.

53. The method of claim 51 wherein,  
said hormones are selected from the group consisting of  
follicle stimulating hormone, luteinizing hormone, chorionic  
gonadotrophin, and gonadotrophin releasing hormone.

5 54. The method of claim 53 wherein,  
said step (a) is accomplished by placing a granulosa cell  
obtained from a preovulatory follicle in said defined culture  
medium.

10 55. A method for assaying the potency of an unknown  
gonadotrophin comprising the steps of:

a) providing an established cell line which secretes  
a known amount of a specified steroid hormone in response to  
contact by a specified amount of a known gonadotrophin, said  
known gonadotrophin having a known biopotency,

15 b) contacting the cells of said cell line with said  
unknown gonadotrophin,

c) determining the quantity of said steroid hormone  
secreted into the medium surrounding said cells, and

20 d) comparing said quantity with said known amount to  
determine the biopotency of said unknown gonadotrophin.

56. The method of claim 55 wherein,

said step (a) is accomplished by employing ovarian  
follicular cells as said established cell line.

25 57. The method of claim 55 wherein,  
step (a) is accomplished by employing human ovarian  
follicular cells.

58. The method of claim 55 wherein,

said step (a) is accomplished by providing a cell line  
which secretes a known amount of progesterone.

30 59. The method of claim 55 wherein,  
said step (a) is accomplished by providing a cell line  
which secretes a known amount of estrogen.

60. A method for determining the toxicity of a test compound comprising the steps of:

- a) providing an established cell line having cells which exhibit a characterized response to a known toxin, said response being a known change in the hormone-secretion profile of said cells of said cell line,
- b) contacting said cells with said test compound,
- c) determining the hormone-secretion profile of said cells after step (b), and
- 10 d) comparing the hormone-secretion profile of said cells after step (b) with said known change in hormone-secretion profile to determine the relative toxicity of said test compound.

61. A cell culture comprising:

- 15 hormone-secreting cells and an establishing medium, said establishing medium comprising:
  - (a) a basal medium having essential minerals, salts, vitamins, amino acids, and lipids,
  - (b) a buffering system,
  - 20 (c) an osmolarity of about 248 mOsm to about 300 mOsm, and
  - (d) glutamine in the amount of about 6.35 mM to about 8.35 mM.

62. The cell culture of claim 61 wherein,  
25 said medium further comprises at least one energy source selected from the group consisting of lactate and pyruvate.

63. The cell culture of claim 61 wherein,  
said medium further comprises serum in the amount of about 0.5% to about 15% of the total volume of the medium.

- 30 64. The cell culture of claim 63 wherein,  
said serum comprises at least one of a human serum and a defined serum supplement.

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65. A cell culture as defined in claim 61 wherein said defined medium includes a protein.
66. The cell culture of claim 65 wherein, said medium further comprises at least one energy source 5 selected from the group consisting of lactate and pyruvate.
67. The cell culture of claim 65 wherein, said osmolarity is about 269 mOsm to about 275 mOsm.
68. The cell culture of claim 61 wherein, said secreted hormone is selected from the group 10 consisting of estrogens, progestins, follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotrophin, thyroxin, and insulin.
69. The cell culture of claim 65 wherein said secreted hormone is selected from the group consisting of estrogens, 15 progestins, follicle-stimulating hormone, luteinizing hormone, human chorionic gonadotrophin, thyroxin, glucagon and insulin.
70. An establishing medium for use in establishing hormone-secreting cells in vitro comprising:
  - (a) a basal medium having essential minerals, salts, 20 vitamins, amino acids, and lipids,
  - (b) a buffering system,
  - (c) an osmolarity of about 248 mOsm to about 300 mOsm, and
  - (d) glutamine in the amount of about 6.35 mM to about 25 8.35 mM.
71. A defined culture medium for use in maintaining hormone-secreting cells in long-term culture comprising:
  - (a) a basal medium having essential minerals, salts, vitamins, amino acids and lipids,
  - (b) a buffering system,
  - (c) protein,

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- (d) an osmolarity of about 248 mOsm to about 300 mOsm, and
- (e) glutamine in an amount of about 6.35 mM to about 8.35 mM.

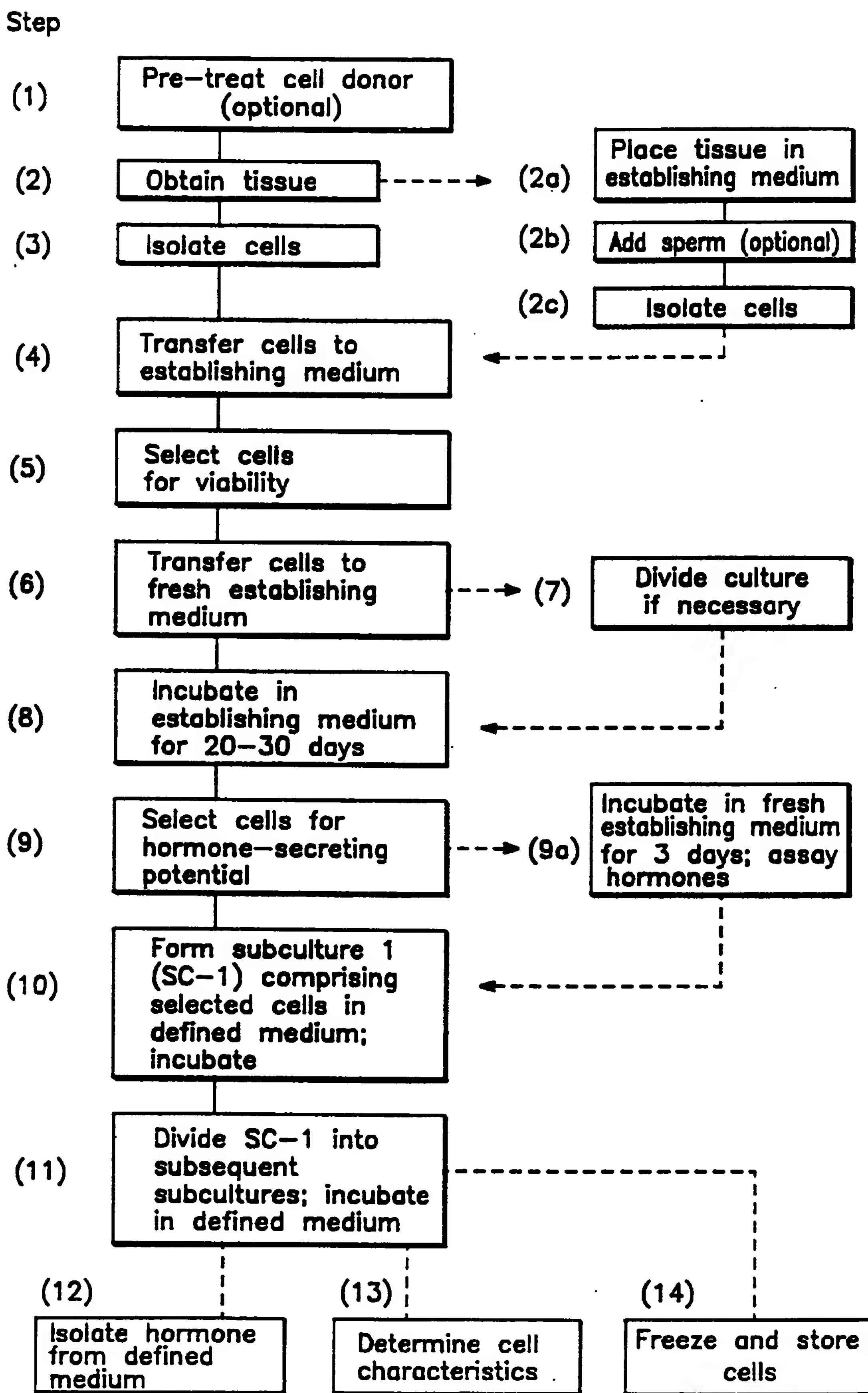


FIG.-1

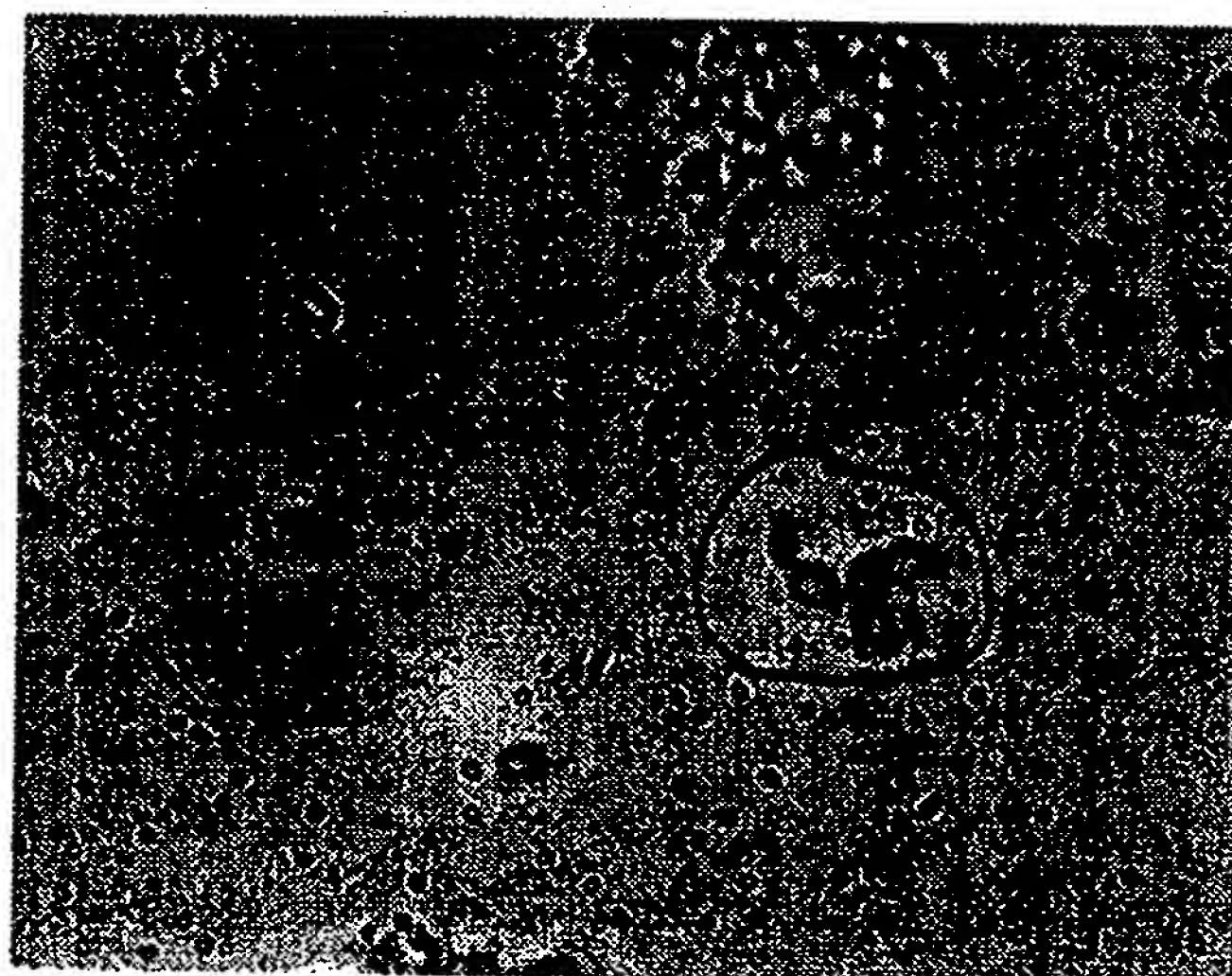


FIG.-2

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FIG.-3

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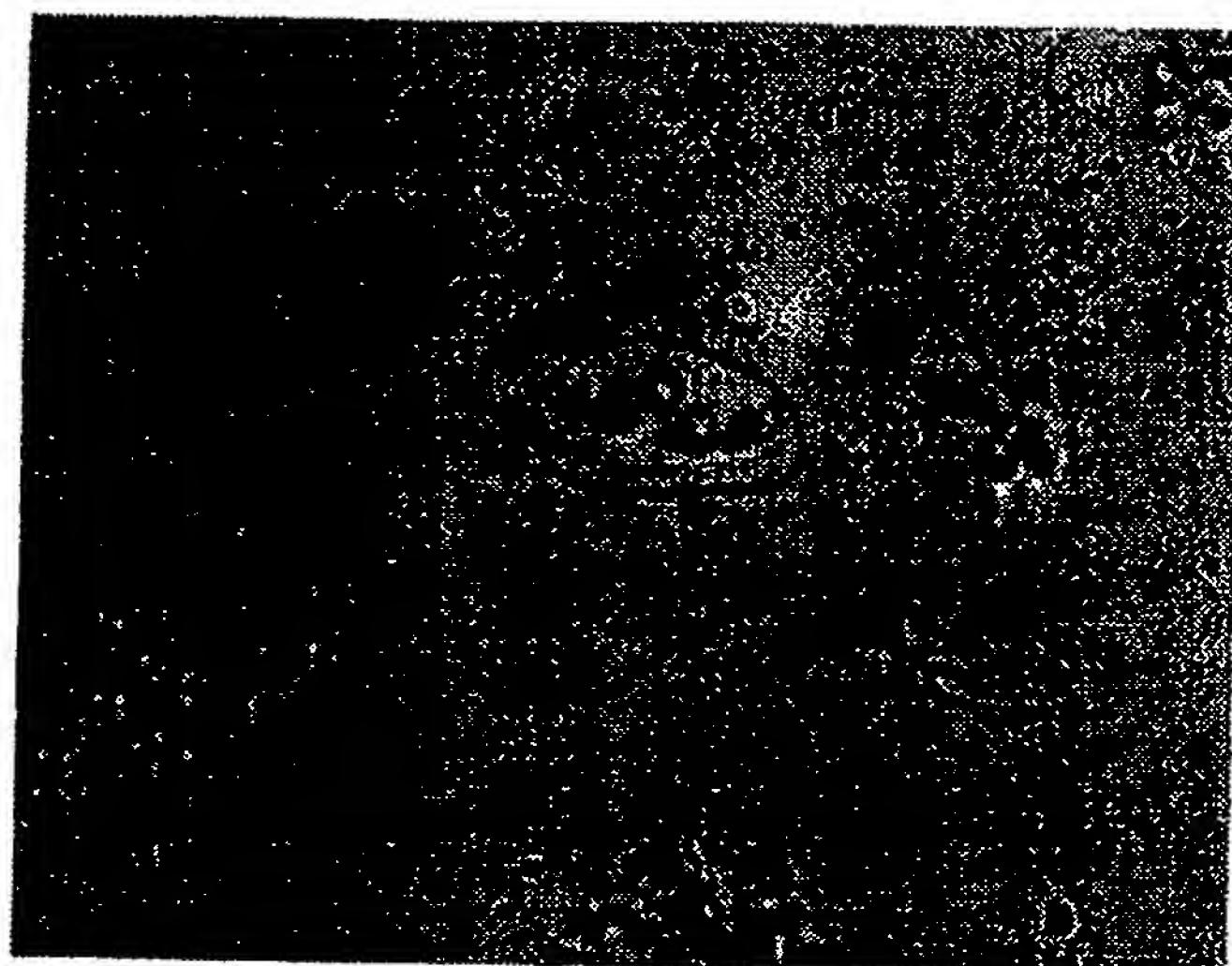


FIG.-4

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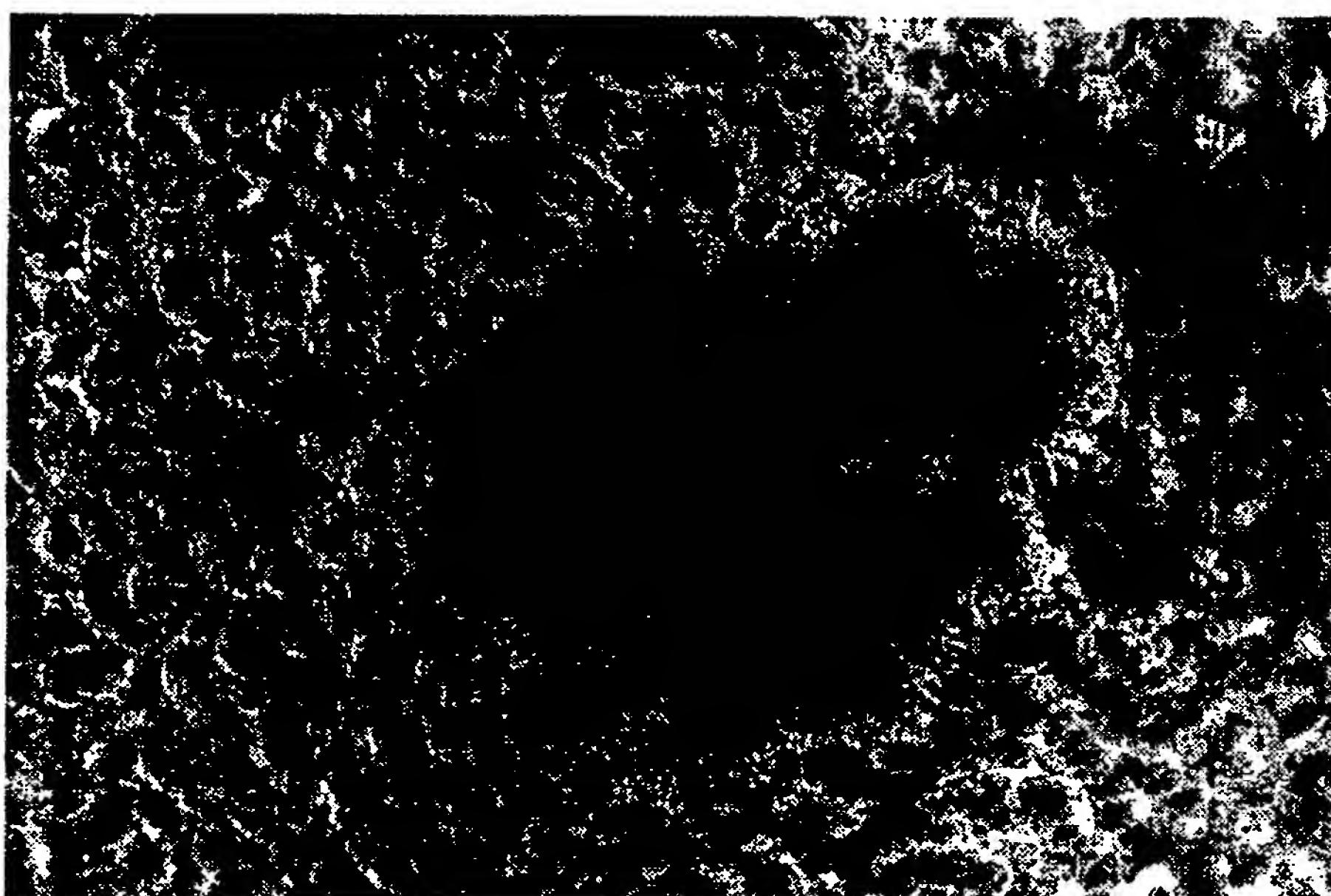


FIG.-5

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FIG.-6

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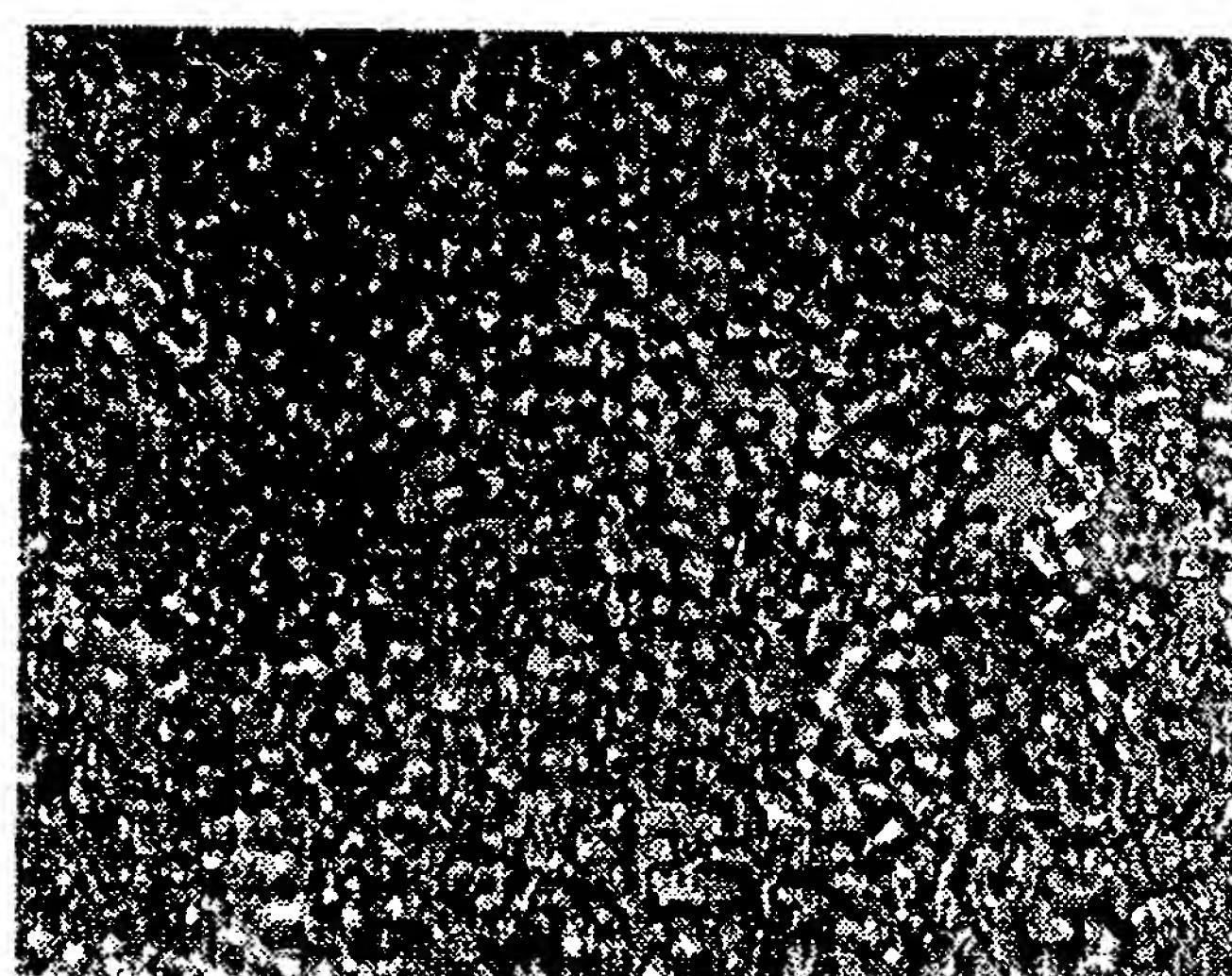


FIG.-7

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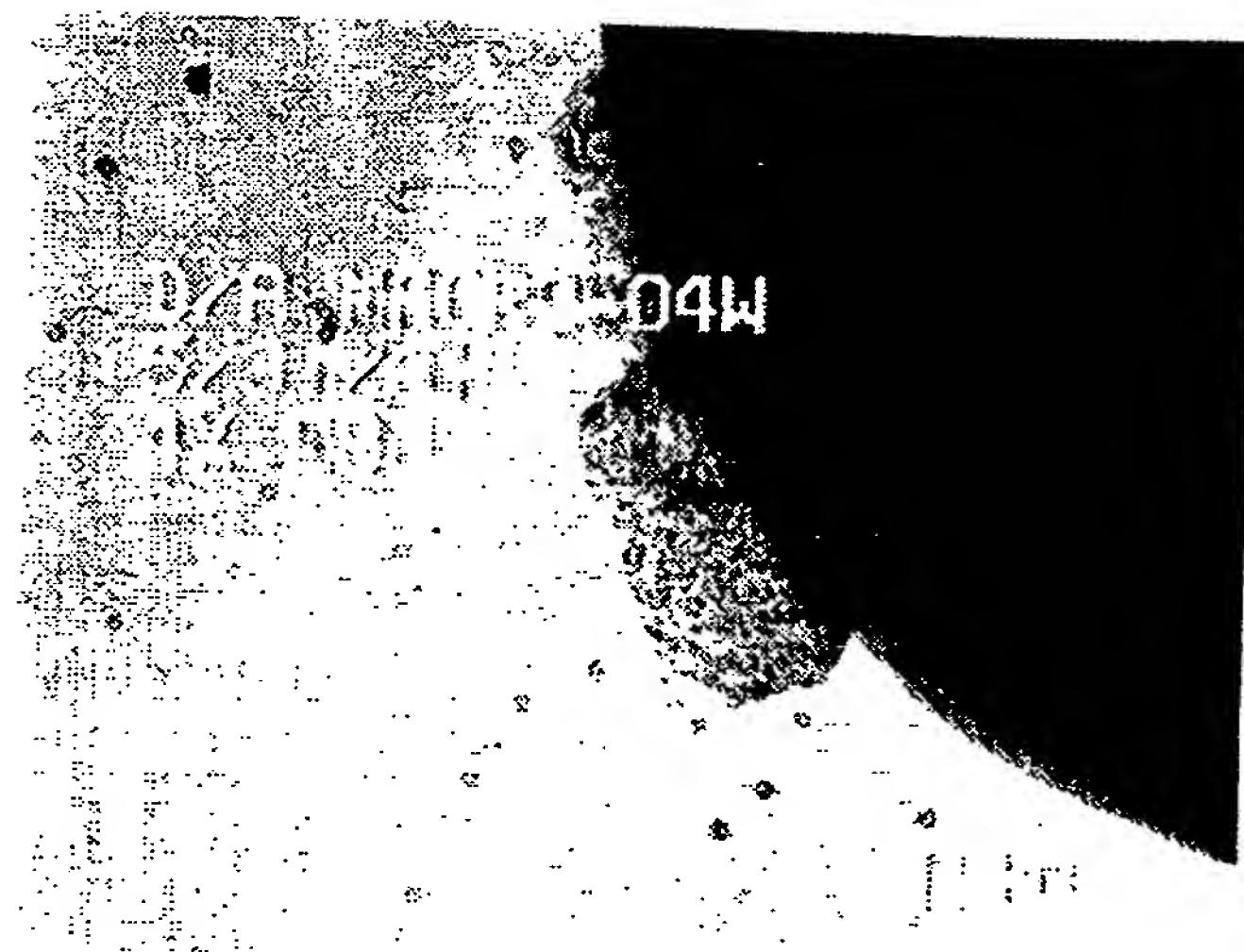


FIG.-8

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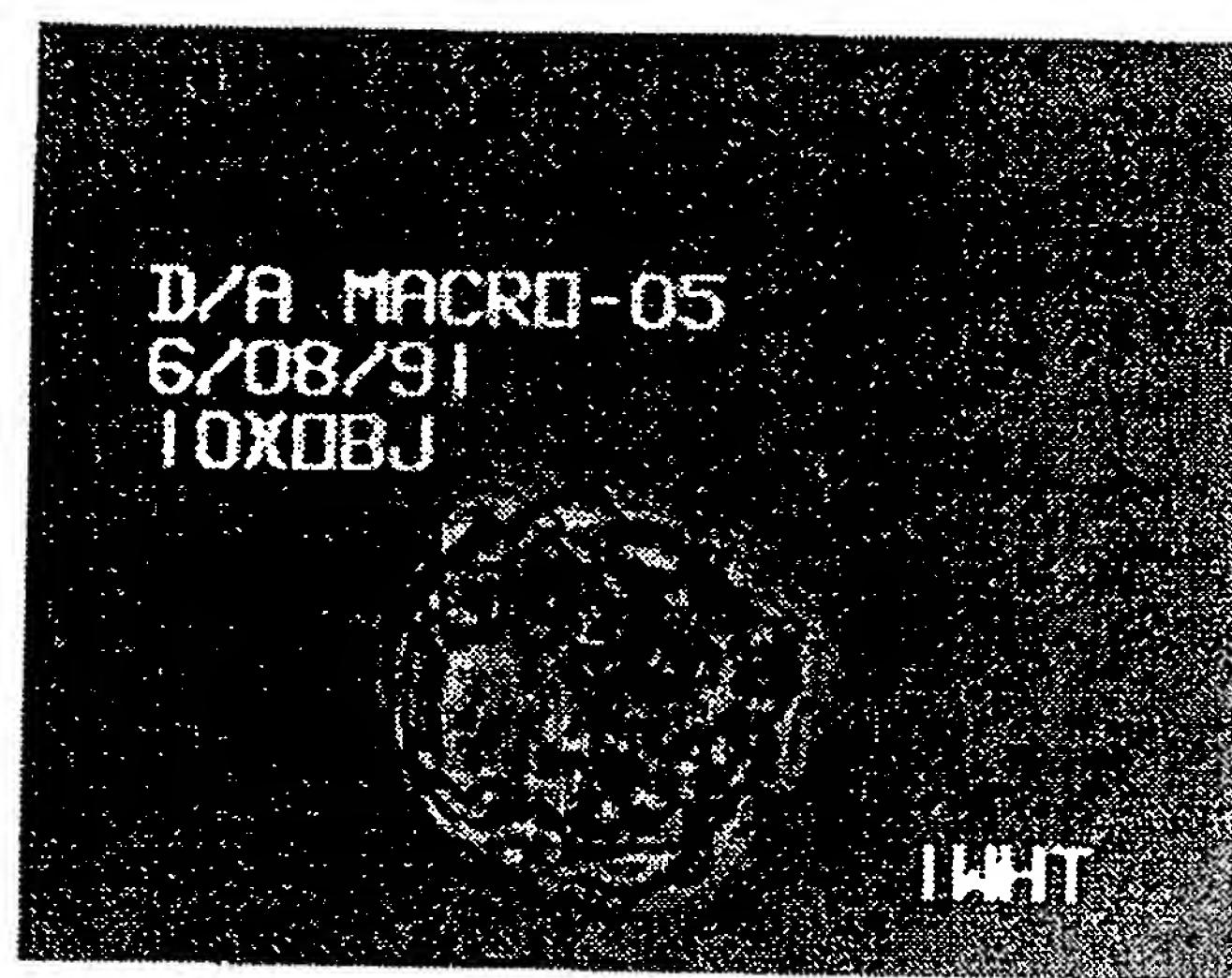


FIG.-9

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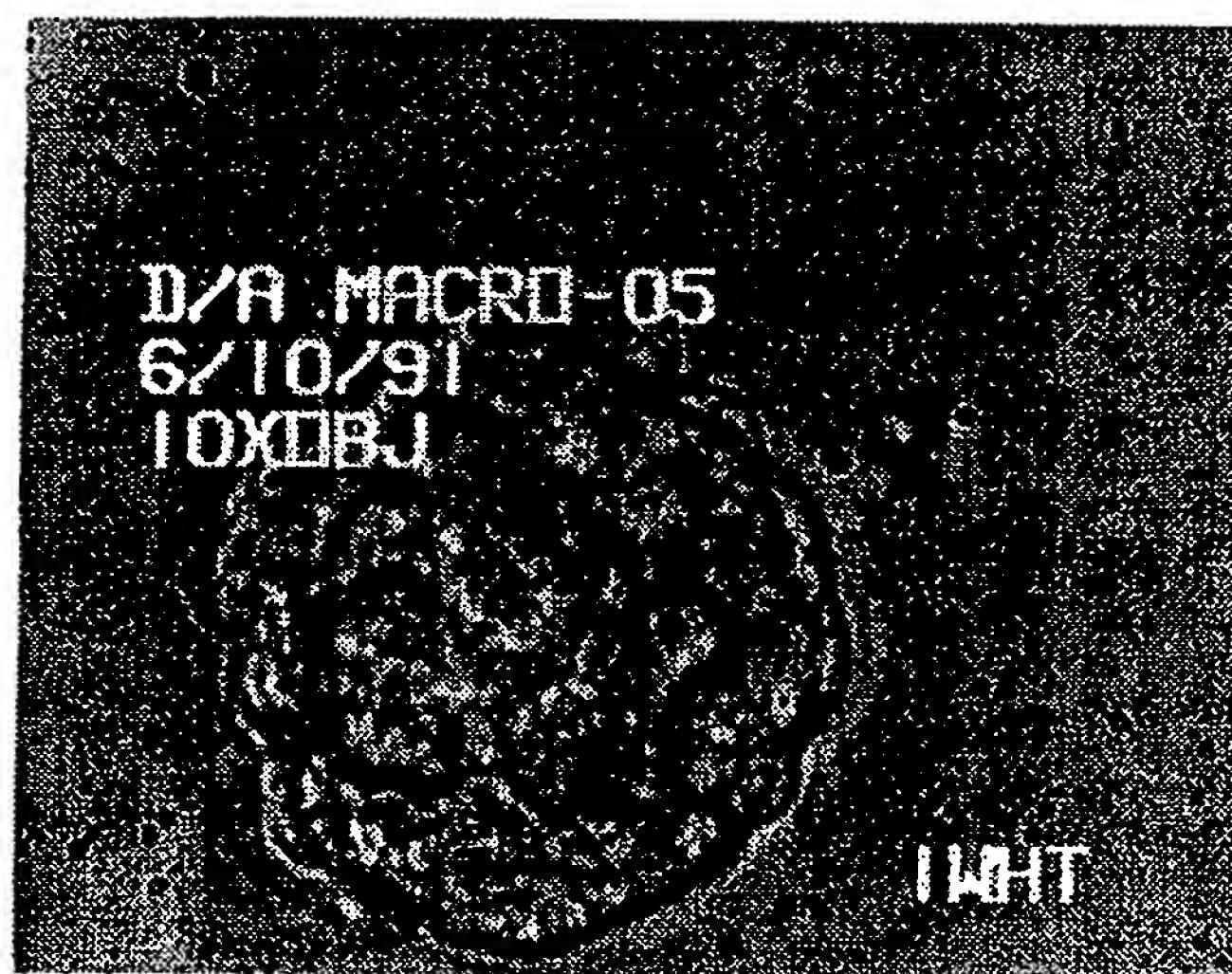
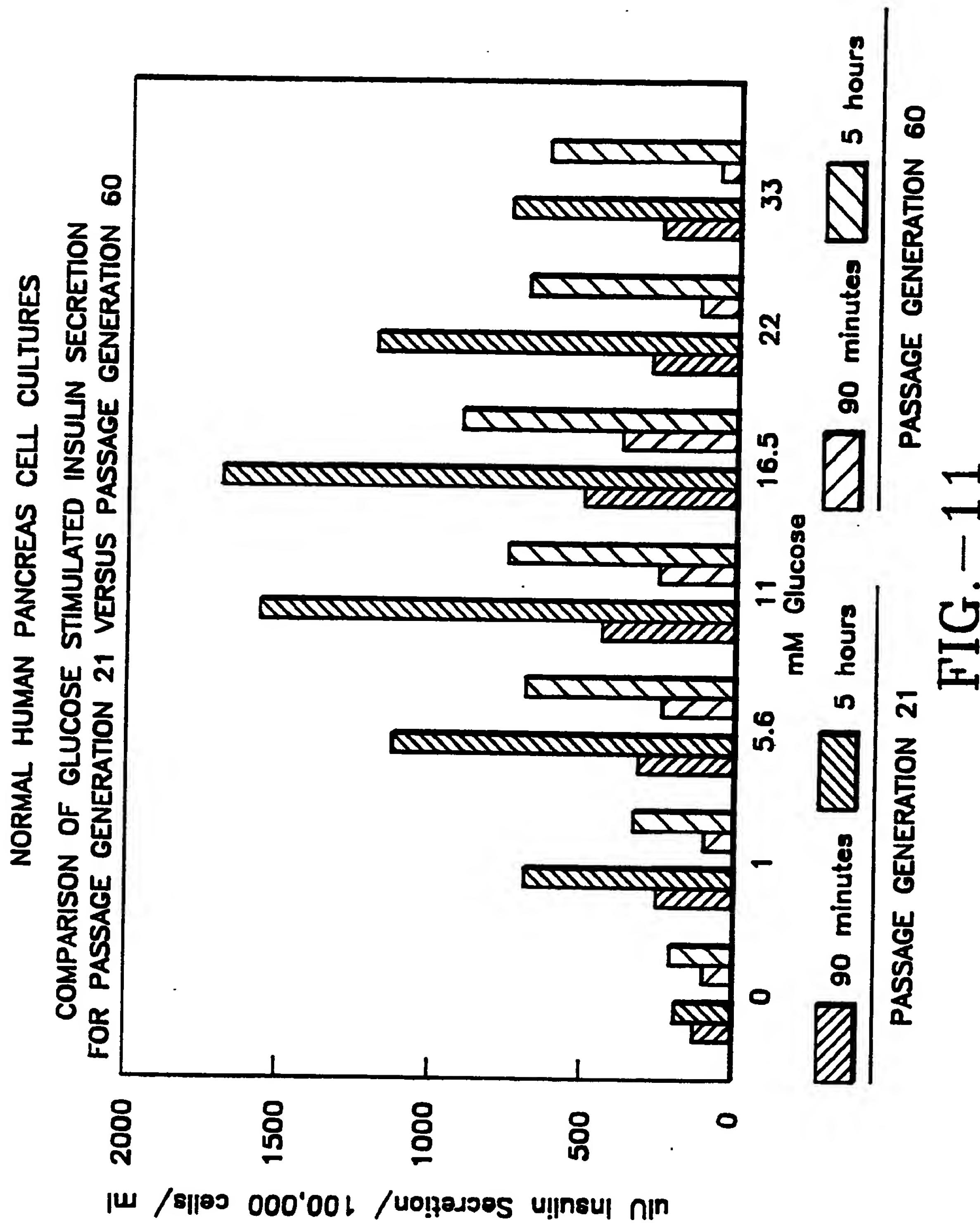


FIG.-10

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## **SUBSTITUTE SHEET**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/05267

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12P 19/00

US CL : 435/72.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/72.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Cancer Res., Vol. 28, issued July 1968, R.A. Patti & G.O. Gey, "The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro", pages 1231-1236, see entire document.	20-42, 61-71
Y	J. Endocr., Vol. 113, issued 1987, K.W. Ng et al., "Insulin release from a cloned precursor beta cell line", pages 3-10, see entire document.	20-42, 48-54
X — Y	Endocrinology, Vol. 125, No. 3, issued 1989, J.L. Tilly & A.L. Johnson, "Regulation of androstenedione production by adenosine 3',5'-monophosphate and phorbol myristate acetate in ovarian thecal cells of the domestic hen", pages 1691-1699, see entire document.	43-54, 60

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

09 SEPTEMBER 1992

Date of mailing of the international search report

5 SEP 1992

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/05267

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Endocrinology, Vol. 124, No. 4, issued 1989, A. Amsterdam et al., "Synergistic effect of human chorionic gonadotropin and extracellular matrix on in vitro differentiation of human granulosa cells: progesterone production and gap junction formation", pages 1956-1964, see entire document.	48-54, 61-71
Y	Endocrinology, Vol. 123, No. 4, issued 1988, J.S. Mondschein et al., "Effects of transforming growth factor- $\beta$ on the production of immunoreactive insulin-like growth factor I and progesterone and on $^3\text{H}$ -thymidine incorporation in porcine granulosa cell cultures", pages 1970-1976, see entire document.	60-71
X — A	In Vitro Cellular & Developmental Biology, Vol. 25, No. 9, issued September 1989, R. Takaki, "Culture of pancreatic islet cells and islet hormone producing cell lines "morphological and functional integrity in culture", pages 763-768, see entire document, especially page 763, line 40.	1-20 — 21-71